AD_____
(Leave blank)

Award Number:

W81XWH-09-2-0081

TITLE:

EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE

PRINCIPAL INVESTIGATOR:

Thomas S. Kilduff, Ph.D.

CONTRACTING ORGANIZATION:

SRI International
Menlo Park, CA 94025-3493

REPORT DATE:

September 2011

TYPE OF REPORT:

Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 July 2011	Annual	1 Sep 2010 – 31 Aug 2011

4. TITLE AND SUBTITLE

EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-2-0081

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Kilduff, Thomas S., Ph.D.

E-Mail: thomas.kilduff@sri.com

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
SRI International
Menlo Park, CA 94025

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

During Year 2, a new laboratory for behavioral performance assessment and microdialysis sampling was occupied and two new HPLCs were purchased and calibrated. Both the benzodiazepine receptor agonist zolpidem (ZOL) and the hypocretin (Hcrt) receptor antagonist almorexant (ALM) induced sleep in rodents. However, ALM did not impair performance in a spatial reference memory test whereas ZOL did. Preliminary results indicate that the wake-active Hcrt neurons could be activated in the presence of ALM but not in the presence of ZOL. In contrast, a sleep-active cortical neuron population was equally activated by ALM and ZOL. ALM caused a significant decrease in basal forebrain (BF) glutamate and concurrently increased BF GABA and adenosine during NREM/REM sleep compared to ZOL or VEH. These results are consistent withthe hypothesis that the disfacilitation of wake-promoting systems by ALM results in less functional impairment than the general inhibition of neural activity produced by ZOL.

15. SUBJECT TERMS
Sleep, performance, drug, neurotransmitter, hypocretin, orexin, benzodiazepine, zolpidem, neurochemistry, microdialysis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	20	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

"Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance"
USAMRAA Grant W81XWH-09-2-0080
DR080789P1
Year 2: 8/1/10 to 7/29/11
Thomas S. Kilduff, Ph.D., Principal Investigator

INTRODUCTION

Almorexant (ALM) is a hypocretin/orexin (Hcrt) receptor antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The overall hypothesis that underlies this research is that ALM produces fewer functional impairments than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. Whereas the human study component will establish if ALM is superior to ZOL in neurocognitive tests, the animal studies will compare the neural circuitry that underlies the activity of these compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Task 2. *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*
2a. Assessment of Almorexant effects on spatial reference memory in rats (months 1 to 12).
2b. Assessment of Almorexant effects on spatial working memory in rats (months 1 to 12).
2c. Assessment of Almorexant effects on psychomotor vigilance in rats (months 13 to 24).
2d. Synthesis of ALM (months 1-4). COMPLETED

Progress – Tasks 2a and 2b: Significant progress has been made on Task 2a. As discussed in last year's progress report, the water maze (WM) and video tracking system was set up in temporary space while our permanent lab was under construction. One group of 24 rats was studied in the temporary space. Following completion of our permanent lab at the end of August, 2010, we moved the WM to room LW103A in this new facility. After extensive testing and revalidation of the experimental setup, all further experiments have been performed in LW103A.

To date, 72 male Sprague-Dawley rats (325-350g) have been implanted with telemetry devices for recording electroencephalograph (EEG), electromyograph (EMG), core body temperature (T_b) and locomotor activity (LMA). Following a minimum of 3 wks for recovery from surgery, each rat was recorded for a 24 h period to assess undisturbed sleep/wake patterns. The test for assessing the effects of Almorexant (ALM) on spatial reference memory in rats occurred on 2 consecutive days. On day 1, the acquisition of the task occurred in one session consisting of 8 consecutive WM trials with a 60 second (s) inter-trial interval. On the following day, rats were dosed 6 h into their active period (ZT18), left undisturbed for 90 min, and then a retention probe trial was performed. For this test, the platform was removed from the WM and the rats were allowed to swim and search for the platform for 30 s. Parameters measured during

the retention probe trial included the time and distance traveled in the quadrant of the WM where the platform had been on the acquisition day, as well as the latency and the number of entries into the target quadrant. EEG and EMG recordings were analyzed from the beginning of lights out (ZT12) until initiation of the WM test (7.5 h later). For more details on our experimental procedures, please see the full protocols in our original proposal.

Results: Both ALM (100 mg/kg i.p and p.o.) and zolpidem (ZOL, 30 mg/kg i.p. and 100 mg/kg p.o.) had significant sleep-promoting effects (Figure 1). Waking (W) was decreased and non-rapid eye movement sleep (NR) increased by ALM and ZOL compared to vehicle. Although NR was increased to a greater extent following ZOL than ALM, rapid eye movement sleep (REM) was increased significantly more by ALM compared to ZOL. Importantly, for the 30 min just prior to WM testing, both the ALM and ZOL groups of rats slept equivalent amounts. The differences between the sleep-promoting effects of ALM and ZOL occurred primarily during the first 30 min following drug administration. Confirming our previous findings, ZOL significantly reduced the latency to sleep onset compared to vehicle and ALM.

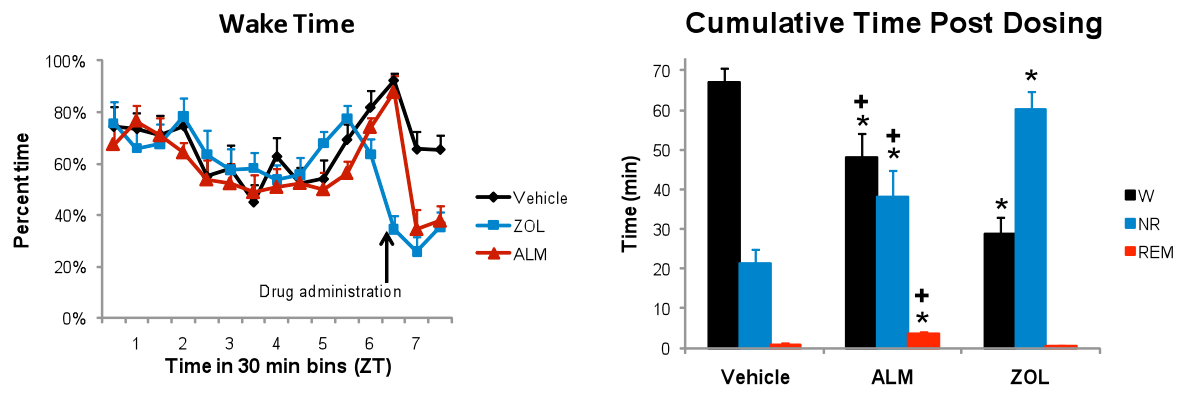

Figure 1. Time spent in W, NR and REM from lights off (ZT12) until the start of the water maze test at ZT19.5. Dosing occurred at ZT18 (6 h after lights off). **Left:** 30 min averages of time spent in W, NR and REM across the recording period. **Right:** cumulative time spent in W, NR and REM following drug treatments until the start of the water maze test. * = significantly different from vehicle; + = significantly different from ZOL.

During the WM probe trial, rats administered ZOL showed impairments in all parameters measured compared to rats administered vehicle or ALM whereas ALM was indistinguishable from vehicle for all measures (Figure 2). Following ZOL, rats swam less, took longer to reach the target zone, spent less time in the target zone and entered the target zone less frequently compared to rats administered vehicle or ALM. These results support our initial hypothesis in which we predicted that rats would perform more poorly following ZOL than following ALM. These results demonstrate that, although ALM is a potent hypnotic, it impairs the performance of rats less than ZOL does in this task.

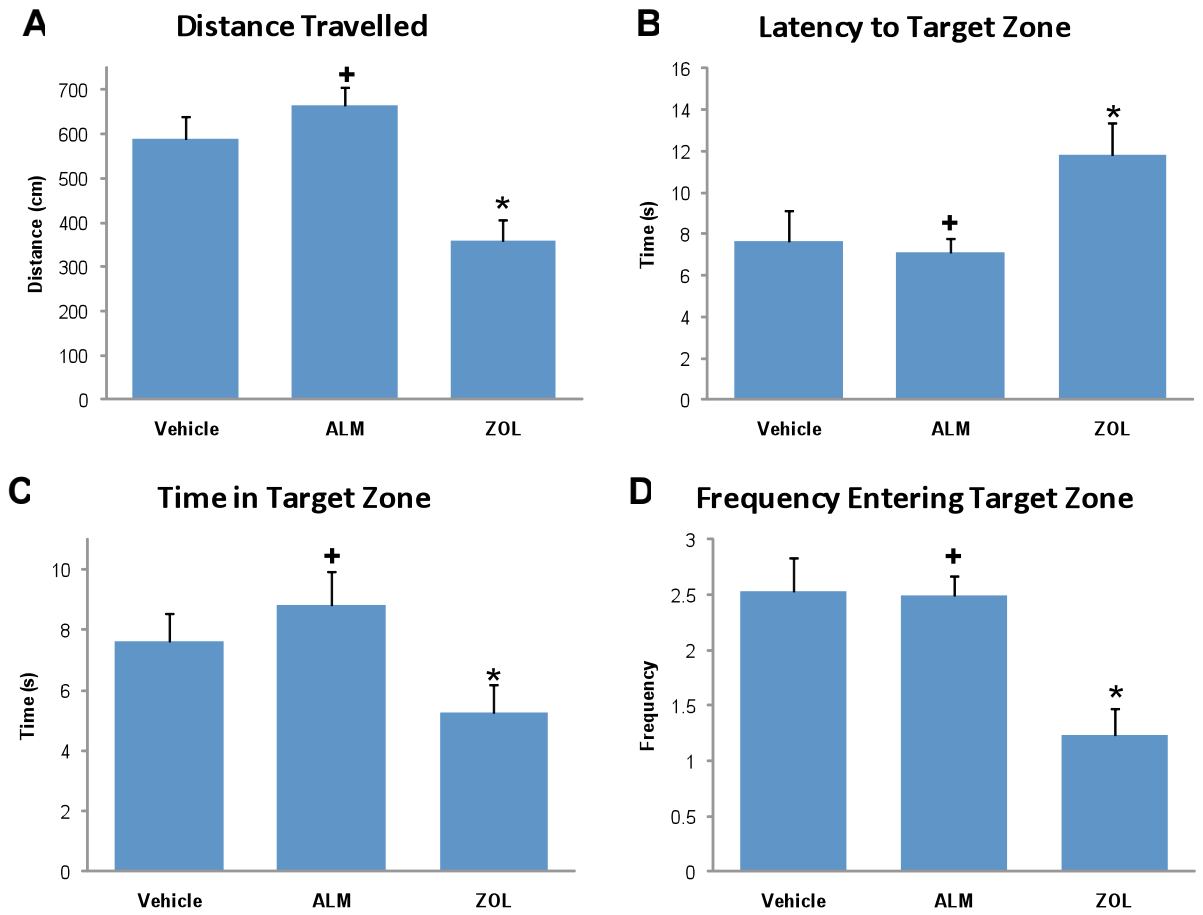

Figure 2. Measures of spatial reference memory during the WM probe trial. Dosing occurred 6 h after lights off and rats were tested 90 min later. **A.** Distance travelled during the 30 s probe trial. Rats administered ZOL swam significantly less than rats administered vehicle or ALM. **B.** Latency to entry into the target zone. Rats administered ZOL took significantly longer time to enter the target zone compared to rats administered vehicle or ALM. **C.** Time spent in the target zone. Rats administered ZOL spent significantly less time in the target zone compared to rats administered vehicle or ALM. **D.** Frequency of entry into the target zone. Rats administered ZOL entered the target zone significantly fewer times compared to rats administered vehicle or ALM. * = significantly different from vehicle; + = significantly different from ZOL.

Task 3. *Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the BzRA ZOL causes a generalized inhibition of the brain.*
3a. Double-label immunohistochemistry with Fos and phenotypic markers (months 1 to 12).
3b. Assessment of hypnotic efficacy in saporin-lesioned rats (months 13 to 24).
3c. Assessment of hypnotic efficacy in transgenic mice (months 25 to 36).

Progress –Task 3a: To assess the influence of ALM and ZOL on the activity of sleep/wake regulatory neurons, we performed an immunohistological study using c-Fos as a marker of neuronal activity. Twenty of the rats prepared for Task 2 above were administered

ALM (100 mg/kg i.p.), ZOL (30 mg/kg i.p.), or vehicle at ZT18 as described above. Half of the animals in each drug treatment condition were allowed to sleep for 1.5h after dosing, whereas the remaining rats were sleep deprived by gentle handling. All animals were then deeply anesthetized, perfused and the brains sectioned. Double-label immunohistochemistry for the marker of functional activity, Fos, and the neuropeptide hypocretin (Hcrt; a "wake-active" hypothalamic neuronal population) was performed in coronal brain sections at the level of the lateral hypothalamus. Analysis of double-labeled neurons revealed that the wake-active Hcrt neurons showed higher levels of Fos expression after SD than after the undisturbed condition only in the ALM-treated rats (Fig. 3) whereas there was no such difference for the ZOL-treated rats. These preliminary results indicate that activation of the Hcrt neurons by SD is unimpaired in the presence of ALM whereas ZOL inhibits this population irrespective of behavioral state. A second batch of tissues from another cohort is currently being processed to increase the sample size for this experiment.

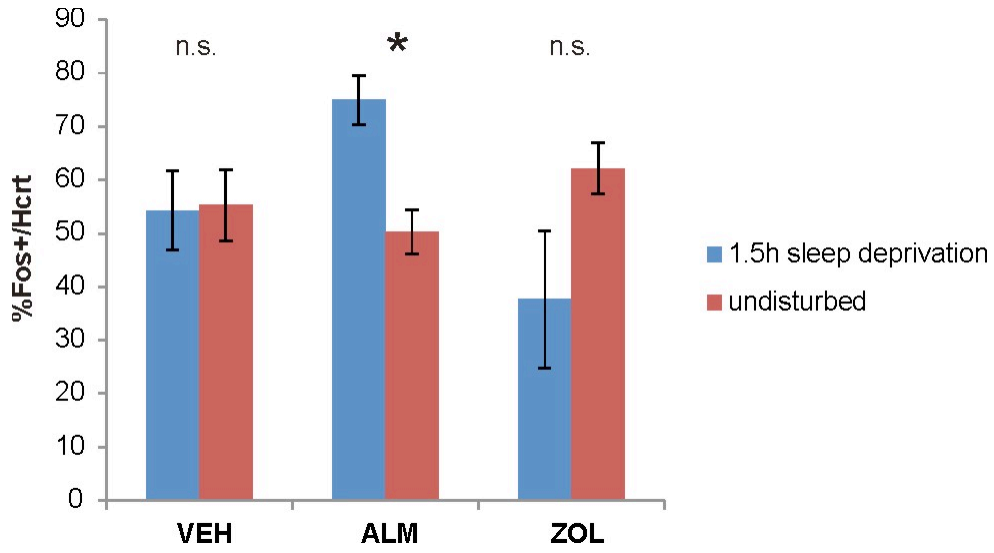

Figure 3. Effect of drug treatment on wakefulness-induced Fos expression in Hcrt neurons. For each drug condition, the SD and undisturbed conditions were compared using the Student's t-test. *, $p < 0.05$; n.s., not significantly different

Task 4. *Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.*
4a. Effects of ALM and ZOL on sleep-active brain areas (months 1 to 12).
4b. BF adenosine (ADO) release in response to oral ALM and ZOL (months 1 to 24).
4c. BF adenosine (ADO) release in response to ALM and ZOL by dialysis (months 25 to 48).

Progress-Task 4a: Using the procedures described under Tasks 2 and 3 above, 22 mice were administered either ALM (100 mg/kg, i.p.), ZOL (30 mg/kg, i.p.) or vehicle. Half of the rats from each drug treatment condition were then either sleep deprived for 1.5h or left undisturbed, followed by perfusion. Double immunohistochemistry for the marker of functional activity, Fos, and the enzyme neuronal nitric oxide synthase (nNOS; a "sleep-active" cortical neuron population) was performed in coronal sections at the level of the anterior commissure. Counts of double- and single-labeled nNOS-immunoreactive cortical neurons revealed that SD inhibited Fos expression in these sleep-active neurons in all drug conditions (Fig. 4). This finding

indicates that cortical nNOS neurons are not activated directly by either ALM or ZOL; rather, their activation is coupled to sleep, which is promoted by both compounds.

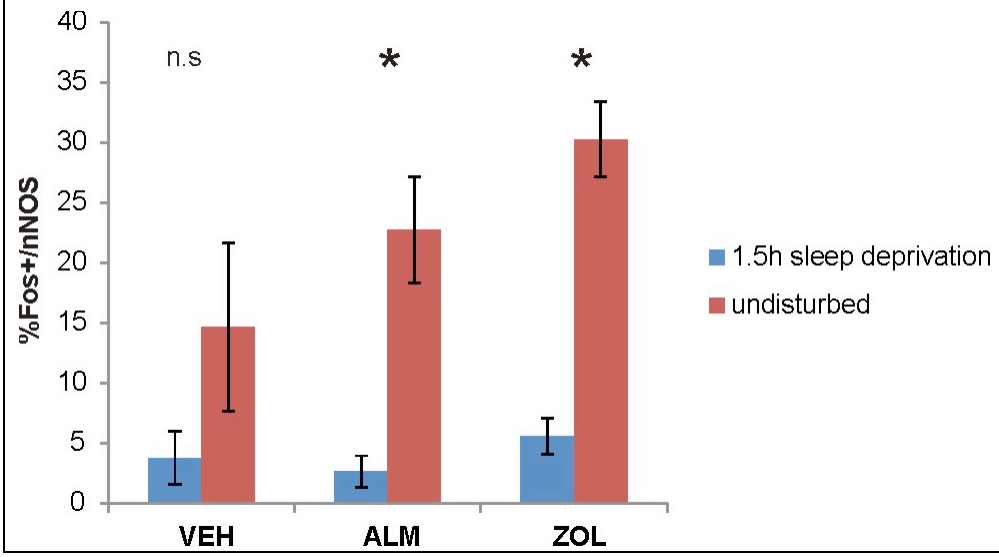

Figure 4. Effect of drug treatment on Fos expression in "sleep-active" cortical nNOS neurons. For each drug condition, the SD and undisturbed conditions were compared using the Student's t-test. *, $p<0.05$, n.s., not significantly different.

Progress – Task 4b: As indicated in the last progress report, experiments and infrastructure crucial to Task 4b and 4c were dependent on the completion of a newly-constructed *in vivo* microdialysis facility. We are pleased to report that excellent progress on Task 4b has been made since the facility was completed in November, 2010. Last year, we summarized progress made on validation of 3 HPLC/EC systems, optimized for catecholamine analyses (System 1: norepinephrine, epinephrine, and dopamine; System 2: serotonin; System 3: acetylcholine). We also received approval to purchase an HPLC for determination of GABA, glutamate, glycine and other amino acids, to have capabilities well beyond the scope of the adenosine measurements proposed as Task 4b and 4c in the SOW. This year, we report the functionality of the adenosine and amino acid/GABA HPLC systems. We set up these new two HPLCs -- one to measure adenosine using UV-VIS detection, and the other to measure amino acids and GABA using electrochemical detection -- each optimized for their specific neurotransmitter capabilities. Our initial efforts were directed to validation of internal standards for each system to determine the lower limit of detection *in vivo.*

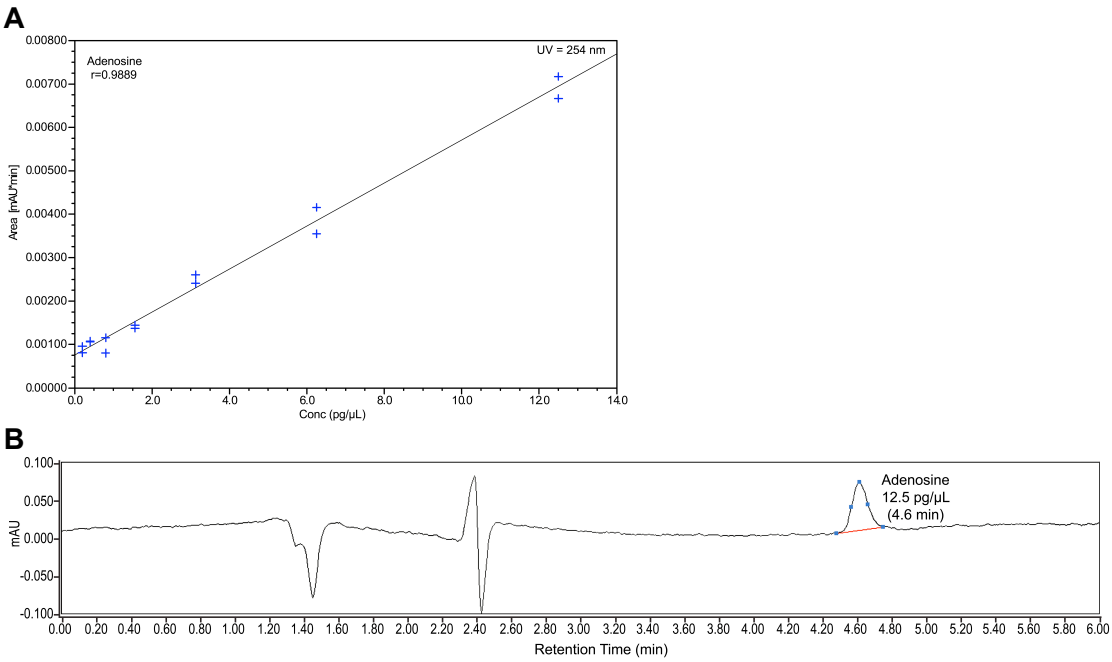

Figure 5. The graph in **A** plots the peak areas detected against the corresponding adenosine external standards (blue crosses). Samples were run in duplicate and averaged peak areas were integrated into a linear fit model to provide the goodness of fit (r value) for adenosine (r=0.9889). The lowest amount of adenosine detected using this calibration curve was 0.2 pg/uL. **B**. Chromatograph showing an individual adenosine peak with its respective concentration and retention time.

For the data presented in Figure 5, samples were injected into the HPLC/UV system (Dionex) for the generation of an external standard curve for adenosine (ADO). Adenosine concentrations were dissolved in oxalic acid (1 mM, pH 3.6), and serially diluted to final concentrations in Ringer's solution. The mobile phase consisted of 10 mM Na_2HPO_4 (pH = 4.5) and 7% acetonitrile. Adenosine was carried through with mobile phase, separated through a Kinetex C18 150 x 4.6mm reversed phase column (Phenomenex) at a flow rate of 0.8 mL/min. UV detection was set to 254 nm. The area under the curve of each peak was measured using Chromeleon 6.8.0 software (Dionex, Corp). Figure 4A plots the peak area detected against the corresponding adenosine external standards (blue crosses). Individual samples of known concentrations were run in duplicate and averaged peak areas were integrated into a linear fit model to calculate the goodness of fit for adenosine (r=0.9889). The lowest limit of detection for adenosine using this calibration curve was 0.2 pg/µL. Figure 5B presents a chromatograph showing an individual adenosine peak with its respective concentration and retention time.

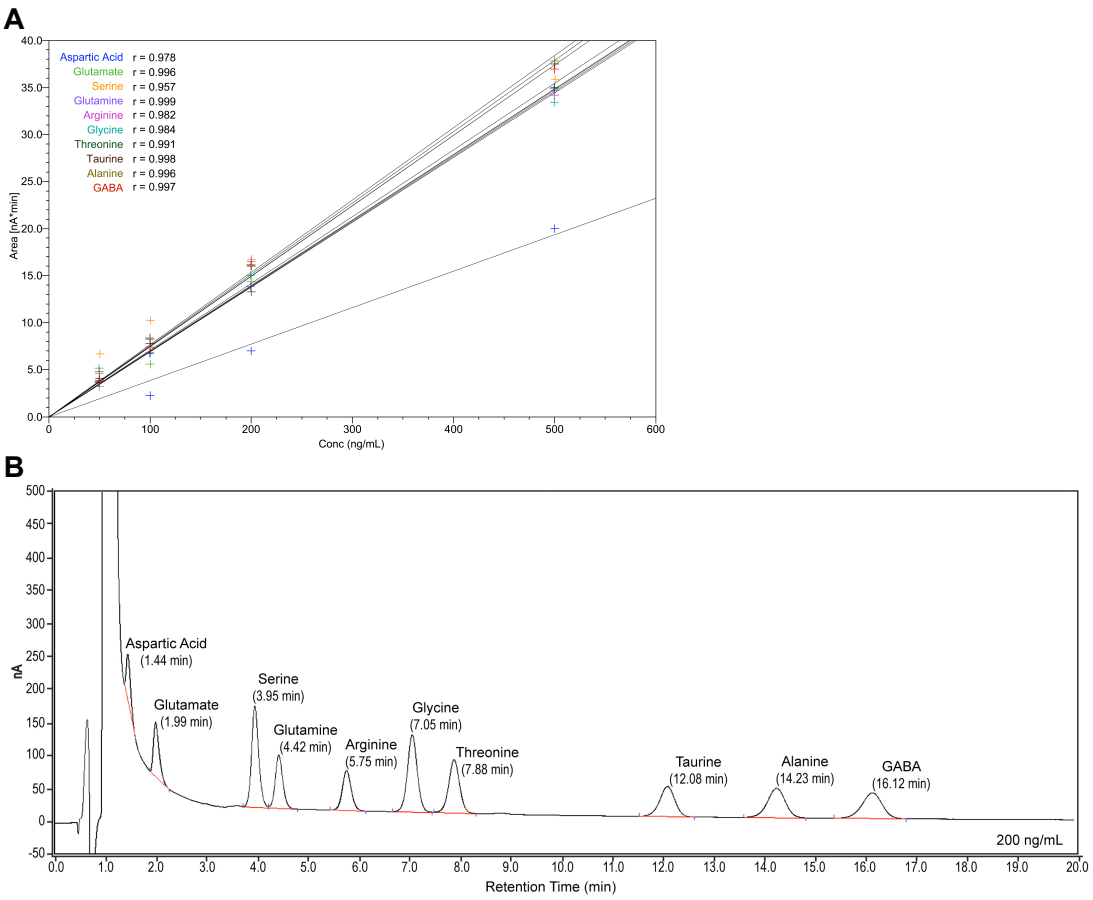

Figure 6. A. Peak areas detected against the corresponding amino acid external standards (Aspartic acid, blue crosses; Glutamate, green; Serine, orange; Glutamine, purple; Arginine, pink; Glycine, light blue; Threonine, dark green; Taurine, brown; Alanine, olive; GABA, red). Samples were run in duplicate and averaged peak areas were integrated into a linear fit model to provide the goodness of fit (r value) for each standard. The lowest amount of neurotransmitter detection using this calibration curve for all amino acids was 5 ng/mL. **B**. Chromatograph showing various amino acid peaks with their respective concentrations and retention times.

For the data presented in Figure 6, individual samples were injected into the HPLC/EC system (Dionex) for the generation of an external standard curve for amino acids and GABA (AA/GABA). Amino acid concentrations were made up as a stock solution, dissolved in 50% methanol (MEOH), and serially diluted to final concentrations in 50% MEOH. The mobile phase consisted of 100 mM Na_2HPO_4, 22% MEOH, and 3.5% acetonitrile, pH 6.75. The amino acid standards were carried through with mobile phase, separated through a Shiseido Capcell Pak C18, 3.0 mm ID x 75 mm, 3 µm reversed phase column from Dionex, and set to a flow rate of 0.4 mL/min. Two electrodes were used, E1; +150 mV, E2; +550 mV, Guard +600 mV. The area under the curve of each peak was measured using Chromeleon 6.8.0 software (Dionex, Corp). Figure 5A plots the peak areas detected against the corresponding amino acid external standards (Aspartic acid, blue crosses; Glutamate, green; Serine, orange; Glutamine, purple; Arginine,

pink; Glycine, light blue; Threonine, dark green; Taurine, brown; Alanine, olive; GABA, red). Individual samples were run in duplicate and averaged peak areas were integrated into a linear fit model to provide the goodness of fit (r value) for each standard. The limit of detection using this calibration curve for all amino acids was 5 ng/mL. Figure 6B presents a chromatograph showing various amino acid peaks with their respective concentrations and retention times.

We also report here on the results of the first cohort of animals in Task 4B, to study the effects of oral ALM and ZOL on basal forebrain (BF) adenosine (ADO) release and, in addition, AA/GABA during sleep and wakefulness. We tested the hypothesis that oral ALM induces sleep by facilitating the mechanisms that underlie the transition to normal sleep. In contrast to ZOL, which affects $GABA_A$ receptors that are widely distributed in the CNS, we hypothesize that ALM acts through blockade of post-synaptic Hcrt receptors, thereby disfacilitating excitation in the BF. We used *in vivo* microdialysis and HPLC analyses to examine BF glutamate, GABA, and ADO efflux following oral ZOL (10 mg/kg), ALM (100 mg/kg), or placebo (VEH) combined with behavioral sleep analyses. Male Sprague-Dawley rats (300 ±25 g) used in this study were housed in an ambient-controlled recording room under a 12 h light/12 h dark cycle (lights off at 04:00) with food and water available *ad libitum*. Room temperature (24±2^{o}C), humidity (50±20% relative humidity), and lighting conditions were monitored continuously via computer. Animals were inspected daily in accordance with AAALAC and SRI guidelines.

Experimental design. Male Sprague-Dawley rats (N=9) were implanted with chronic recording devices (F40-EET, Data Sciences Inc., St Paul, MN) for continuous recordings of electroencephalograph (EEG), electromyograph (EMG), core body temperature (T_{core}), and LMA via telemetry as described previously (Morairty et al., 2008). Rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) for surgical implantation of a unilateral, stainless steel 26-gauge guide cannula aimed at the BF for microdialysis recovery of ADO, glutamate, and GABA. BF coordinates relative to bregma were P -0.3, L +2.0, V –5.0 (Paxinos and Watson, 2009). The electrodes, guide cannula (to permit microdialysis probe placement in the brain of freely-moving rats) and a stainless steel skull screw were fixed to the skull using dental cement. A dummy probe was inserted into the cannula to prevent occlusion prior to the onset of dialysis. For microdialysis, the dummy probe was removed and the microdialysis probe was inserted and locked into position such that the tip of the probe membrane extended 2.0 mm below the edge of the guide cannula.

Rats were given a 2-3 wk post-surgical recovery period prior to entering the experimental paradigm, conditioned to the microdialysis chamber, and handled for at least 30 min every day for 1 wk prior to the onset of the experiment to limit stress on the day of the experiment from exposure to a novel environment. Animals were also given two separate 1 ml doses of vehicle on two separate days at least one week before the first experimental day. A microdialysis probe was inserted through the guide cannula 16 h prior to the onset of the experiment day and continuously perfused with aCSF. At the start of the experiment (4.5 hours into the dark period, ZT16.5), three 30 min baseline samples (1 µL/min flow rate, 30 µL TV) were collected from freely-moving animals to assess basal levels of ADO, glutamate, and GABA and baseline EEG, EMG, T_b and LMA were collected to assess behavior. Telemetry data were recorded using DQ ART 3.1 software (Data Sciences Inc., St Paul, MN). Each rat received one treatment in random order (washout period minimum 1 week) with parallel microdialysis sampling of the BF. Drug doses included ALM (100 mg/kg), ZOL (10 mg/kg) and VEH. One of three drugs was subsequently given p.o. to the animals 6h into the dark period (the rats' normal active period) (ZT18), and six additional 30 min samples were collected to assess the effects of the drug on behavior and

neurotransmitter release in the BF. Behavioral measures were simultaneously collected for an additional 1.5 h (total 12 h) post-microdialysis. All samples were collected at 4ºC and immediately stored at -80ºC until processed for ADO by HPLC/UV and AA/GABA by HPLC-EC detection.

Behavioral data analyses. Following completion of data collection, sleep-wakefulness was scored in 10 s epochs by examining the recordings visually using Neuroscore software (Data Sciences Inc., St Paul, MN). Any epochs that contained recording artifacts were tagged and excluded from subsequent analyses. EEG and EMG data were scored for waking (W), rapid eye movement sleep (REM), and non-REM (NR). T_{core} and LMA (counts per minute) were analyzed as hourly means. Individual state data were analyzed as time spent in each state (W, REM, and NR) per hour. Latency to NR and REM onset for each rat was calculated from the time of drug injection. To assess any pharmacological effects on the consolidation of behavioral states, cumulative time spent in W, NR, and REM and the duration and number of bouts of each state was calculated for 3 h following drug administration relative to each 30 min dialysis sample obtained pre- and post-drug administration. Descriptive statistics and analysis of variance (ANOVA) analyses were performed on all behavioral measures. Where ANOVA indicated a probability (*P*) value < 0.05, Dunnett's *post hoc* was used to determine significance between groups.

HPLC analyses. All microdialysis samples were split (10 µL for ADO, 20 µL for AA/GABA) into two vials for HPLC analyses. ADO samples were separated by reverse-phase HPLC with a Kinetic column (Phenomenex C18 150 x 4.6mm) and monitored at 254 nM by UV. The mobile phase consisted of 10 mM Na_2HPO_4 (pH = 4.5), and 7% acetonitrile and was set to a flow rate of 0.8 mL/min. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex, Corp). Amino acids, glutamate and GABA were assayed using HPLC-EC. The mobile phase consisted of 100 mM Na_2HPO_4, 22% MEOH, and 3.5% acetonitrile, pH 6.75 and set to a flow rate of 0.4 mL/min. The amino acids were detected by precolumn derivitization using O-phtaladehyde (OPA) and 2-mercaptoethanol (βME) with automation at 4°C, 2 min prior to injection into the HPLC. Separation was achieved with a reversed-phase column by Shiseido (Capcell Pak C18, 3.0 mm ID x 75 mm, 3 µm) and electrically detected at the following potentials; E1; +150 mV, E2; +550 mV, Guard +600 mV. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex Corp). Descriptive statistics and a two-way ANOVA were used to determine the effect of sleep-wake states on ADO, glutamate, and GABA release. Post hoc comparisons were performed using Tukey's multiple pairwise comparison tests. A probability (*P*) value < 0.05 was used to evaluate the significance of all statistical tests.

Behavioral State Results. To date, a total of 9 rats contributed to the current set of results. As illustrated in Figure 7, representative hypnograms show the effects of VEH, ZOL (10 mg/kg, p.o.), and ALM (100 mg/kg, p.o.) administration on individual animals' sleep-wake architecture for each treatment condition.

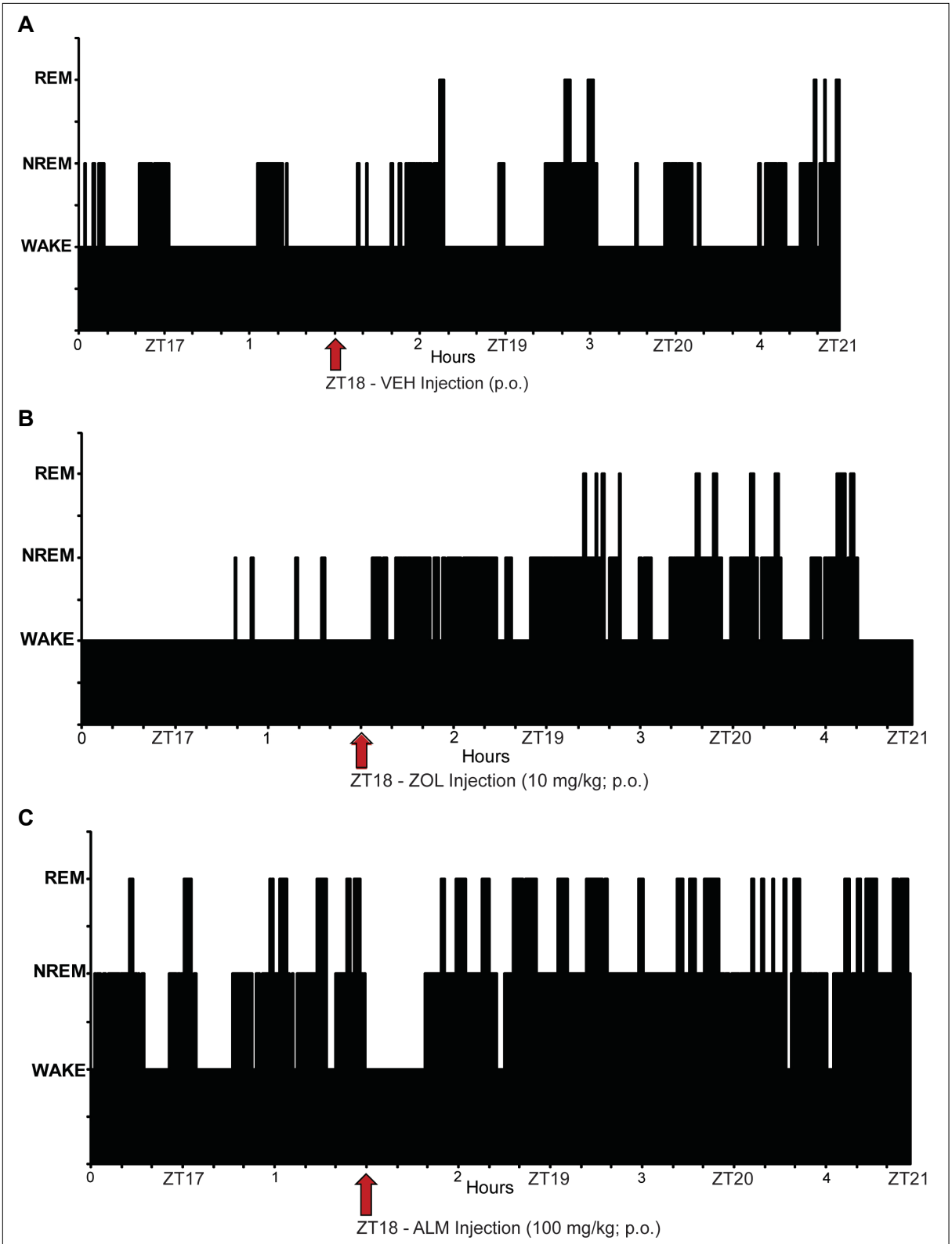

Figure 7. Individual sleep-wake hypnograms showing the effects of each drug treatment on sleep and wakefulness over time. **A**. Hypnogram of a rat injected with vehicle (VEH) at ZT18 and resultant sleep-wake pattern. **B**. Sleep–wake architecture of a rat injected (ZT18) with ZOL. **C**. Sleep-wake pattern of a rat animal injected (ZT18) with ALM.

Table 1 summarizes the effects of VEH, ZOL, and ALM administration (p.o.) on sleep-wake parameters for each treatment condition following drug delivery. ANOVA revealed a significant drug effect on wake, NR, and R states (*$p<0.05$). Dunnett's *post-hoc* analyses showed that ZOL and ALM had significant effects on the total amount of time spent in Wake and NR compared to VEH. In addition, ALM significantly increased (*$p<0.05$) mean Wake duration, and the number of NR and R bouts compared to VEH control animals.

Table 1. Sleep-wake parameters in VEH, ZOL-, and ALM- treated rats post-drug administration. Values (means ± SEM) are calculated for a 3 h period during the dark phase.

Sleep/Wake Parameter	VEH	ZOL (10 mg/kg)	ALM (100 mg/kg)
Cumulative Wake (min)	144.2 (11.6)	**96.2 (9.3)***	**61.7 (5.2)***
No. of Wake Bouts	20.0 (4.2)	22.2 (2.5)	25.4 (1.5)
Mean Duration of Wake Bouts (min)	8.3 (2.8)	4.6 (0.8)	**2.4 (0.1)***
Cumulative NREM (min)	33.8 (9.3)	**75.4 (7.6)***	**87.7 (2.6)***
No. of NREM Bouts	20.3 (4.4)	25.6 (2.7)	**38.0 (2.3)***
Mean Duration of NREM Bouts (min)	1.6 (0.3)	**2.9 (0.2)***	2.3 (0.2)
Latency to NREM (min)	9.6 (5.8)	9.6 (3.8)	17.0 (2.0)
Cumulative REM (min)	4.94 (2.5)	11.2 (2.1)	**33.5 (3.5)***
No. of REM Bouts	5.0 (2.6)	10.0 (2.3)	**24.2 (3.2)***
Mean Duration of REM Bouts (min)	0.7 (0.3)	1.3 (0.3)	1.4 (0.2)
Latency to REM (min)	22.3 (11.5)	44.6 (13.4)	26.2 (1.2)

*$p<0.05$ determined by 1-way ANOVA with Dunnett's multiple comparison *post hoc* analyses.

Neurotransmitter Release Results. The results to date are based on a total of 3,390 min of microdialysis sampling across the sleep-wake cycle from 9 rats. Dialysis samples were split into two and processed for both ADO and glutamate/GABA content. Two-way ANOVA revealed a significant drug x state interaction for all neurotransmitters. Tukey's *post hoc* comparisons showed that oral ZOL (*$p<0.05$) caused a significant increase in BF glutamate release (Figure 8A) when sampling during 30 min timeframes comprised of wakefulness compared to ALM or VEH. Oral ALM (*$p<0.05$) significantly increased BF glutamate (Figure 8B) under sampling conditions that showed a mixture of Wake, NR, and R. On the other hand, ALM decreased BF glutamate (Figure 8C) during sampling when only NREM/REM sleep occurred during the compared to ZOL. Since no 30 min consolidated NREM/REM period occurred following VEH, the comparison between VEH and drug treatments could not be made.

Oral ALM concurrently decreased BF GABA release as demonstrated by Tukey's post-hoc (*$p<0.05$) during dialysis sampling periods when the animals cycled between Wake, NR, and R (Figure 9B). On the other hand, GABA release was significantly higher in the ALM (*$p<0.05$) condition compared to ZOL during NREM/REM cycling and the corresponding collection timeframes (Figure 9C).

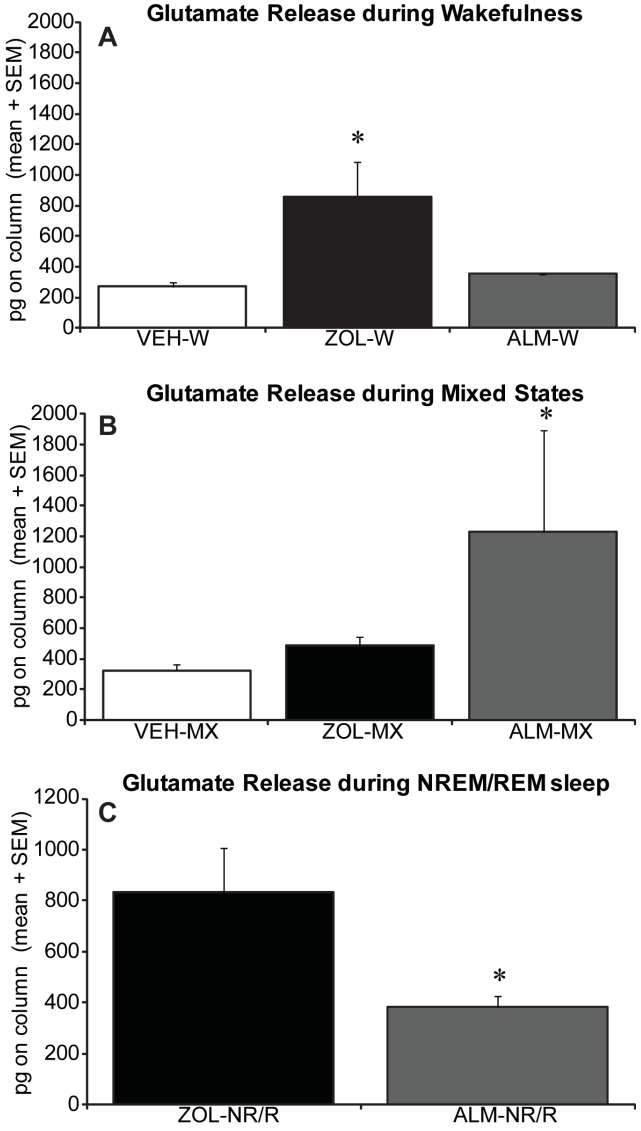

Figure 8. Glutamate release changes during sleep-wakefulness as a function of drug treatment. **A**. Tukey's *post-hoc* comparisons showed that ZOL increased glutamate release when sampling during states of wakefulness (*p<0.05) compared to ALM. **B**. Glutamate release under sampling conditions that showed a mixture of Wake, NREM (NR), and REM (R), was significantly higher in the ALM (*p<0.05) condition compared to ZOL. **C**. ALM caused a significant decrease in BF glutamate during NR/R and the corresponding collection timeframes compared to ZOL or VEH (*p<0.05).

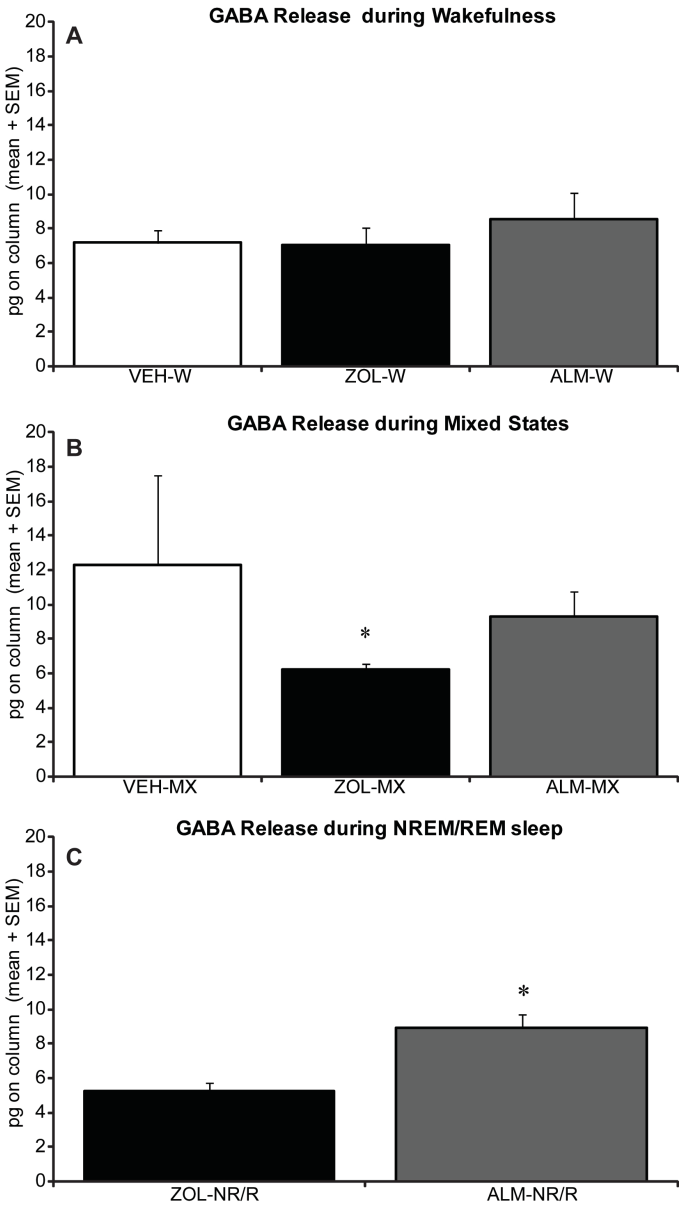

Figure 9. GABA release changes during sleep-wakefulness as a function of drug treatment. In **B**, *post hoc* analyses showed that ZOL decreased GABA release during sampling while the animals presented mixed states of Wake, NR, and R. **C**. GABA release was significantly higher in the ALM condition compared to ZOL during NREM/REM cycling and the corresponding collection timeframes (*p<0.05).

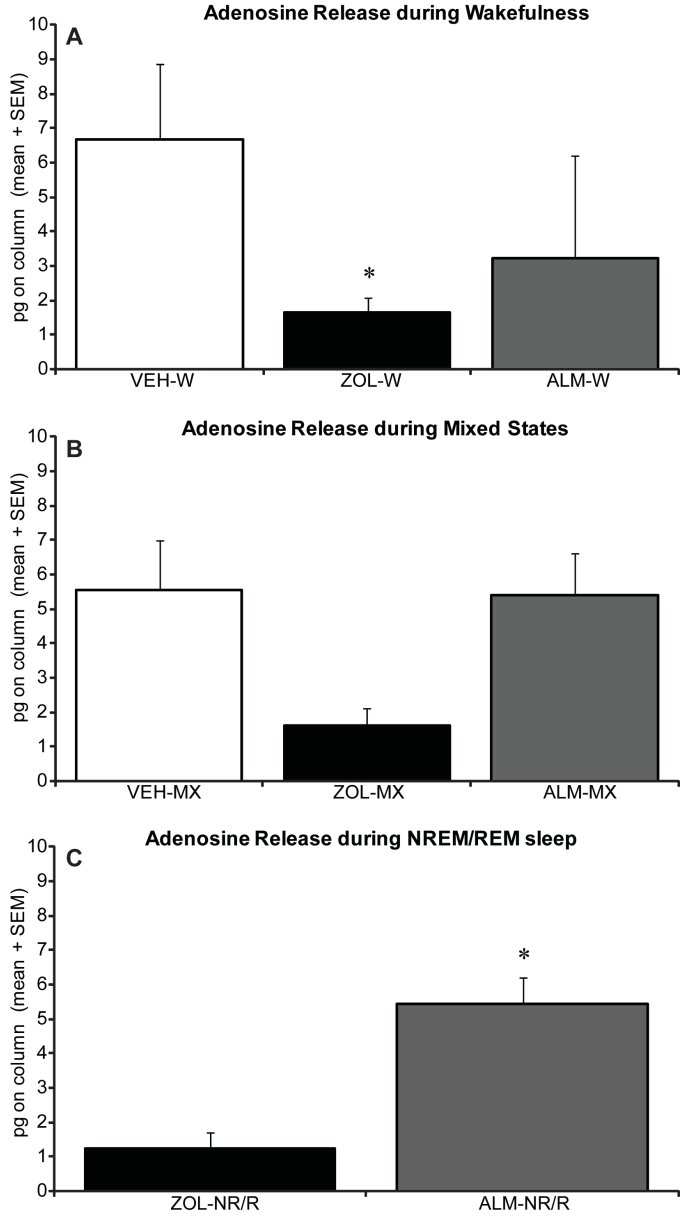

Figure 10. ADO release changes during sleep-wakefulness as a function of drug treatment. **A**) Post-hoc analyses showed that ZOL decreased ADO release (*p<0.05) when sampling during states of wakefulness. **C**) ADO release was significantly higher in the ALM condition compared to ZOL during NREM/REM cycling (*p<0.05).

Analyses of ADO levels revealed that oral ZOL caused a significant decrease in BF ADO release (Figure 10A) when sampling during 30 min timeframes comprised of wakefulness compared to ALM or VEH (*p<0.05; Tukey's *post hoc* test). However, oral ALM significantly increased BF ADO (Figure 10C; *p<0.05) under sampling conditions where only NREM/REM cycling occurred during the corresponding collection timeframes compared to ZOL or VEH. These neurotransmitter results provide additional evidence for dynamic neurochemical changes underlying Hcrt modulation of sleep-wakefulness.

Task 5: *Test the hypothesis that neural gene expression that occurs ALM-induced sleep more closely resembles that of spontaneous sleep than does ZOL-induced sleep.*
5a. Comparison of ALM and ZOL effects on expression of plasticity-related genes (months 37 to 48).
5b. Comparison of ALM and ZOL effects on brain gene expression in comparison to spontaneous sleep (months 37 to 48).

Progress: None anticipated prior to Year 3.

Plans for Year 3:

Task 2: Tasks 2a and 2b will be completed. For Task 2c, the assay will be established and data collection will begin. The order for the testing chamber will be placed in August, 2011.

Task 3: Immunostaining of brains for other wake-active neuronal populations (cholinergic, serotonergic and noradrenergic neurons) is ongoing. Michael Schwartz, Ph.D. will join our staff as a Research Scientist on August 1, 2011 to lead the efforts on Task 3b in Year 3. He will also initiate acquisition and breeding of mice as necessary to execute Task 3c, which will likely occur primarily in Year 4.

Task 4: We will continue collecting data from another cohort of animals in order to reach our proposed statistical power of 8 rats per treatment group in Task 4b. The behavioral and neurotransmitter analyses will then be submitted for publication. The study design for Task 4b, BF ADO release in response ALM and ZOL by local dialysis will require some experimental modifications as we have determined that neither ALM nor ZOL readily pass across the dialysis membrane. The drug characteristics and permeation rate of ALM and ZOL depend upon the concentration of drug, the oil/water partition coefficient of drug, and the surface area of the dialysis membrane. To permit sufficient compound of either ALM or ZOL to flow across the membrane, we've surmised that (1) neither ALM nor ZOL would have relative efficacy on release or behavior at the minimum concentration, (2) these compounds are not readily dissolved in water, and (3) the surface area of the dialysis membrane is limited by the area of the brain structure (small region within the BF) in which we want the drugs to freely diffuse across without affecting other major brain regions. Thus, we propose to microinject ALM and ZOL (using several different concentrations) into the BF and collect microdialysis samples from the sleep-active cortical regions (Gerashchenko et al, 2008) of freely-moving animals instead and assess ADO, glutamate and GABA release along with simultaneous behavioral measures.

Task 5: As follow up to Dr. Kilduff's July 22, 2011 email and July 25, 2011 letter to LCDR Mark D. Clayton, Ph.D., CDMRP Science Officer, we request permission to modify the focus of Task 5 from gene expression studies to optogenetics and *in vivo* cellular neurophysiology, which we believe would be more informative technical approaches with regard to the overall theme of "Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance." As is evident in this report, our research efforts involve systems physiology/pharmacology approaches to understand how the Hcrt receptor antagonist ALM is producing sleep without (apparently) impairing performance. To advance understanding of this question, we believe that optogenetic and *in vivo* cellular neurophysiology approaches would complement our ongoing behavioral, *in vivo* physiology, functional neuroanatomical and neurochemical approaches. In particular, the recent paper published in *Journal of Neuroscience* by Tsunematsu et al. (*J. Neurosci.* 2011; 31:10529-10539) presents a paradox with respect to

understanding how ALM induces sleep since, in that study, optogenetic silencing of the Hcrt cells induces sleep only during the day but not at night. Conversely, previous studies have shown that optogenetic activation of the Hcrt cells through the blue light-sensitive channelrhodopsin (ChR2) protein during sleep reduces the latency to awakening (Adamantidis et al., 2007; Carter et al., 2009).

Using a combination of optogenetics and *in vivo* cellular neurophysiology, we propose to address the following questions related to the overall theme of "Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance":

1) **Is activation of the Hcrt system sufficient to induce arousal in the presence of ALM?** As is evident in Figure 2, ALM-treated rats are able to arouse from ALM-induced sleep and perform at a high level, at least in this spatial reference memory task. The mechanism underlying the arousal from sleep is unlikely to be mediated through the Hcrt peptides because Hcrt receptors should be blocked by the presence of ALM. However, Hcrt neurons also release glutamate and perhaps other unknown neurotransmitters. To address this question, we will induce arousal from spontaneous vs. ALM-induced sleep by optogenetic stimulation of the Hcrt neurons in a transgenic mouse that specifically expresses the blue light-sensitive ChR2 in Hcrt neurons (*orexin/ChR2* mice). If the Hcrt system is truly involved in arousal from sleep, ALM should block optogenetic-induced awakenings. If optogenetic-induced awakenings persist in the presence of ALM, we can conclude that other neurotransmitters released by the Hcrt neurons are the likely cause of the arousal. These data will complement the behavioral studies being conducted in Task 2.

2) **How does ALM affect the activity of subcortical sites downstream from the Hcrt neurons?** Although the optogenetic papers cited above as well as others in the literature review in our original proposal focus on the importance of the Hcrt neurons for maintenance of wakefulness and arousal from sleep, Hcrt neurons are part of a larger network that underlie sleep/wake control. To this point, there is no information of the effects of ALM on these "downstream" Hcrt efferent projection sites. We hypothesize that ALM will blunt optogenetically-induced neural activation in subcortical Hcrt projection sites such as the serotonergic dorsal raphe nucleus (DR) and noradrenergic locus coeruleus (LC) whereas ZOL will not. To address this hypothesis, we will conduct optogenetic stimulation of Hcrt neurons in *orexin/ChR2* mice while simultaneously conducting multiunit recordings from efferent projection sites such as the DR and LC. These data will complement the functional neuroanatomical studies being conducted in Task 3.

3) **How does Hcrt neural activity affect cortical function?** In order to appropriately perform cognitive tasks, the cerebral cortex must be engaged and unimpaired. The data in Figure 2 indicate that performance on a hippocampal-mediated spatial reference memory task is unimpaired in the presence of ALM. Task 2c is intended to compare cortical function in the presence of ALM vs. ZOL. Although both Hcrt receptors 1 and 2 are differentially expressed at low levels within the cortex (Marcus et al., 2001), to this point, it is unknown whether ALM affects cortical neuron activity. We hypothesize that ALM will directly affect the activity of a subset of cortical interneurons present in cortical layers 5 and 6. To address this hypothesis, we will compare the effects of ALM and ZOL on cortical firing and local field potentials using a 64 channel cortical array and a 16 channel vertical probe during the sleep/wake cycle and during optogenetic manipulation of Hcrt neurons. We expect that ZOL will have a generalized inhibitory effect on cortical neuron activity whereas ALM will only affect a subset of cortical

neurons. The results of these studies will assist in the interpretation of the behavioral studies being conducted in Task 2c, the functional neuroanatomical studies in Task 4a and the revised microdialysis studies in Task 4c.

Budgetary implications: Implementation of the optogenetic and *in vivo* cellular neurophysiology approaches proposed here in lieu of the microarray approaches previously proposed will have budgetary implications. Task 5 as originally proposed was scheduled to start in Year 4 whereas we would like to initiate the revised Task 5 in Year 3. In a 24 June 2011 email, Ms. Jennifer Shankle, USAMRAA Grant Specialist, pointed out that we have been underexpending funds on USAMRAA Grant W81XWH-09-2-0081 to date. As explained in Dr. Kilduff's 27 June 2011 response, this underexpenditure was a result of the delay in initiating Tasks during Year 1 while laboratory construction was ongoing. SRI requests approval to spend our current funding to initiate work on the revised Task 5 during Year 3. The funding we currently have available is adequate to complete the currently approved Tasks and to initiate Task 5 in Year 3 and we currently have trained staff on hand who can conduct the proposed studies. We will need to reallocate funds within our current budget as these new approaches will necessitate the purchase of some equipment. Because of the synergism that we expect to result from implementation of these approaches with the approaches used in execution of Tasks 2-4, we believe that this budget reallocation is not only highly justified in terms of achieving the overall goals of this proposal, but that integration of these technical approaches will enable us to conduct higher quality science.

KEY RESEARCH ACCOMPLISHMENTS

• Occupation of a new laboratory for behavioral performance assessment and microdialysis sampling in November, 2010.

• Full system installation of all hardware equipment and software, and validated communication and automation capabilities for two HPLCs: one to measure adenosine and the other to measure amino acids.

• Establishment of a spatial reference memory test and demonstration that ZOL impairs performance on this test whereas ALM does not (Figure 2).

• Preliminary results obtained indicating that the wake-active Hcrt neurons could be activated in the presence of ALM but not in the presence of ZOL (Figure 3).

• Determination that both ALM and ZOL activated a sleep-active cortical neuron population (Figure 4).

• Establishment of limits of detection for the two new HPLCs (Figures 5-6).

• Determination of the effect of oral ALM vs. ZOL on neurotransmitter release in the Sprague-Dawley rat (Figures 8-10).

• Submission of two abstracts to be presented at the annual Society for Neuroscience meeting to be held in Washington, D.C. in Nov 2011.

REPORTABLE OUTCOMES

Abstracts submitted for Society for Neuroscience meeting (Washington, DC, Nov 12-16, 2011)

• L Dittrich, S Morairty, D Warrier, A Wilk, K Silveira, TS Kilduff, "The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake".

• J. Vazquez-DeRose, A. Nguyen, and T. S. Kilduff. "Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat".

CONCLUSION

Preclinical data indicate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. In Year 2, we have found that both the benzodiazepine receptor agonist ZOL and the Hcrt receptor antagonist almorexant (ALM) induced sleep in rodents but ALM did not impair performance in a spatial reference memory test whereas ZOL did. The lack of impairment may, in part, be due to the fact that the wake-active Hcrt neurons could be activated in the presence of ALM but not in the presence of ZOL. In contrast, a sleep-active cortical neuron population was equally activated by ALM and ZOL. During NREM/REM sleep, ALM caused a significant decrease in basal forebrain (BF) glutamate and concurrently increased BF GABA and adenosine compared to ZOL or VEH. These results are consistent with the hypothesis that disfacilitation of wake-promoting systems by ALM results in less functional impairment than the general inhibition of neural activity produced by ZOL.

REFERENCES

Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L (2007) Neural substrates of awakening probed with optogenetic control of hypocretin neurons. *Nature* 450:420-424.

Carter ME, Adamantidis A, Ohtsu H, Deisseroth K, de Lecea L (2009) Sleep homeostasis modulates hypocretin-mediated sleep-to-wake transitions. *J Neurosci* 29:10939-10949.

Gerashchenko, D. Wisor, J. P. Burns, D. Reh, R. K. Shiromani, P. J. Sakurai, T. de la Iglesia, H. O. and Kilduff, T. S. (2008). Identification of a population of sleep-active cerebral cortex neurons. *Proc Natl Acad Sci U S A*, 105(29):10227-32.

Marcus JN, Aschkenasi CJ, Lee CE, Chemelli RM, Saper CB, Yanagisawa M, Elmquist JK (2001) Differential expression of orexin receptors 1 and 2 in the rat brain. *J Comp Neurol* 435:6-25.

Morairty, S. L. Hedley, J. Flores, R. Martin and T. S. Kilduff (2008). Selective $5HT_{2A}$ and $5HT_6$ receptor antagonists promote sleep in rats. *Sleep* 31:34-44.

APPENDICES

None.